FORM PTO-1390 (REV. 12-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: 65959/16

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/049804

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/07736	August 9, 2000	August 16, 1999

TITLE OF INVENTION: PREPARATION OF METAL OXIDE SUPPORTS LOADED WITH BIOMOLECULES

APPLICANT(S) FOR DO/EO/US: F. Venema

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [X] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [X] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information:
    - paper copy of sequence listing;
    - statement that sequence listing adds no new matter; and
    - statement that paper and CRF forms of sequence listing are identical.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/049804	PCT/EP00/07736	65959/16

21. [X] The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00	
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	22 - 20 =	2	x **$18.00**	$ 36.00
Independent claims	3 - 3 =	0	x **$84.00**	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$
TOTAL OF ABOVE CALCULATIONS =				$
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$
SUBTOTAL =				$ 926.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$ 926.00
			Amount to be refunded:	$
			charged:	$

a. [X] A check in the amount of $ 926.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 01-1785 . A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Craig J. Arnold, Esq.
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New York, NY 10016

SIGNATURE

Elie H. Gendloff
NAME

44,704
REGISTRATION NUMBER

AR&E Docket No. 65959/16

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	F. Venema
Serial No.	:	10/049,804
I.A. Filing Date	:	February 14, 2002
For	:	PREPARATION OF METAL OXIDE SUPPORTS
	:	LOADED WITH BIOMOLECULES
Examiner	:	Unknown
Group Art Unit	:	Unknown

SUBMISSION OF CORRECTED SEQUENCE LISTING

U.S. Patent and Trademark Office
Box SEQUENCE
Room 1B03-Mailroom
Crystal Plaza Two
2011 South Clark Place
Arlington, VA 22202

"Express Mail" mailing label No. EL 777785128 US
Date of Deposit: July 26, 2002
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner for Patents, Washington, D.C. 20231.
Name: Elie H. Gendloff
Signature:

Sir:

Enclosed herewith is a corrected Sequence Listing in both paper and computer-readable form for the above-identified application. This submission was required in the Notification of Missing Requirements Under 35 U.S.C. 371 in the United States Designated/Elected Office (DO/EO/US) dated April 29, 2002 (copy enclosed) in the above-identified case and is being filed concurrently with a reply to that Notification, along with a request for one-month extension of time and a check for the appropriate fees.

208691.1

I hereby state that the information recorded in computer readable form is identical to the paper form of the Sequence Listing. Additionally, this Sequence Listing includes no new matter. Entry of the Sequence Listing is therefore respectfully requested.

Respectfully submitted,

AMSTER, ROTHSTEIN & EBENSTEIN
Attorneys for Applicant
90 Park Avenue
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(212) 697-5995

Dated: New York, New York
July 26, 2002

By: _____
Elie H. Gendloff
Registration No.: 44,704

65959/16

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	F. Venema
Serial No.	:	Unknown
Filed	:	Herewith
For	:	PREPARATION OF METAL OXIDE SUPPORTS LOADED WITH BIOMOLECULES
Examiner	:	Unknown
Group Art Unit	:	Unknown

PRELIMINARY AMENDMENT ACCOMPANYING FILING UNDER 35 U.S.C. 371

Commissioner for Patents
Washington, D.C. 20231

Sir:

The following amendments accompany the U.S. national stage filing of PCT/EP00/07736. The amendments to claims 1-2 were originally submitted under PCT Article 19, and the amendments to claims 16 and 17, together with the addition of claims 20-22, are submitted to eliminate the multiple dependencies of claims 3, 16 and 17, and the improper "use" claim of claim 16. The multiple dependency of claim 3 was added as an Article 19 amendment. Claim 3 is also amended to recite a preferred acidic solution.

Applicants also provide herewith a sequence listing, in both computer readable and paper form. The enclosed sequence listing does not go beyond the original disclosure in the international application as filed. Also, the information recorded in computer readable form is identical to the written sequence listing.

Amendment

IN THE SPECIFICATION:

Please add the following paragraph on page 1, immediately after the title.

--This application claims priority to PCT/EP00/07736, filed August 9, 2000, which claims priority to European Patent Application 99202649.2, filed August 16, 1999.--

Please amend claims 1-3, 16 and 17 to read as follows.

1. (Amended) A method for removing non-loaded amino groups which form part of the silanating agent used to activate a metal oxide surface during the preparation of metal oxide supports loaded with biomolecules, comprising the steps of:

(a) activating the surface of the support by means of a silanating agent comprising an amine group;

(b) loading the support by attaching biomolecules to the activated surface; and

(c) treating said loaded support with an acidic solution.

2. (Amended) A method for removing non-loaded amino groups which form part of the silanating agent used to activate a metal oxide surface during the preparation of metal oxide supports loaded with biomolecules, comprising the steps of:

(a) activating the surface of the support by means of a silanating agent comprising an amine group;

(b) loading the support by attaching biomolecules to the activated surface; and

(c) treating said loaded support with a basic or neutral solution.

3. (Amended) The method of claim 1, wherein the solution is of pH 2 to 6.

16. (Amended) The metal oxide support of claim 14, suitable for performing a probe-based assay.

17. (Amended) A kit comprising the metal oxide support of claim 14, further comprising a detection means for determining whether binding has occurred between the biomolecules and an analyte.

Please add the following new claims 20-22.

20. (New) The method of claim 2, wherein the solution is pH 6-7.

21. (New) The metal oxide support of claim 15, suitable for performing a probe-based assay.

22. (New) A kit comprising the metal oxide support of claim 15, further comprising a detection means for determining whether binding has occurred between the biomolecules and an analyte.

Remarks

This application has 22 claims, including amended claims 1-3, 16 and 17, and new claims 20-22. The claim amendments are solely to make the claims more consistent with U.S. practice. As such, the claims as amended are fully supported by the specification as originally filed. Additionally, any narrowing claim amendments made herewith are not made for purposes of patentability, but rather to make the claims consistent with U.S. patent practice and to eliminate multiple dependencies to avoid the

fees for filing multiple dependent claims.

The specification is also amended to provide priority information in the first paragraph of the application, as required under U.S. patent law.

Respectfully submitted,

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Dated: New York, New York
February 14, 2002

By: _____
Elie H. Gendloff
Registration No.: 44,704

Appendix - Marked up Claim Amendments
U.S. National Stage of PCT/EP00/07736
Additions are underlined and deletions are bracketed.

1. (Amended) A method for removing non-loaded amino groups which form part of the silanating agent used to activate a metal oxide surface during the preparation of metal oxide supports loaded with biomolecules, comprising the steps of:

(a) activating the surface of the support by means of a silanating agent comprising an amine group;

(b) loading the support by attaching biomolecules to the activated surface; and

(c) treating said [characterized in that subsequently the]loaded support [is treated]with an acidic solution[and provided that the method is not used for the preparation of silica wafers which are aminated by silanation using (3-aminopropyl)monoethoxydimethylsilane and loaded with oligonucleotides].

2. (Amended) A method for removing non-loaded amino groups which form part of the silanating agent used to activate a metal oxide surface during the preparation of metal oxide supports loaded with biomolecules, comprising the steps of:

(a) activating the surface of the support by means of a silanating agent comprising an amine group;

(b) loading the support by attaching biomolecules to the activated surface[,]; and

(c) treating said [characterized in that subsequently the]loaded support [is treated]with a basic or neutral solution[and provided that the method is not used for derivatization of alunimiumoxide nanoparticles aminated with (3-aminopropyl)triethoxysilane, wherein the basic solution further contains a large excess of N-acetylhomocysteinelactone].

3. (Twice Amended) The method of claim 1[or 2], wherein the solution is of pH 2 to 6[7].

16. (Amended) [Use of the]The metal oxide support of claim 14[or 15], suitable for performing a probe-based assay.

17. (Amended) A kit [of parts]comprising the metal oxide support of claim 14[or 15], further comprising a detection means for determining whether binding has occurred between the biomolecules and an analyte.

Untitled.ST25.txt
SEQUENCE LISTING

<110> PAMGENE B.V.

<120> Preparation of metal oxide supports loaded with biomolecules

<130> PAM-003-PCT

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PREPARATION OF METAL OXIDE SUPPORTS LOADED WITH BIOMOLECULES

The invention relates to a method for the preparation of metal oxide supports loaded with biomolecules, to the thus obtained metal oxide supports, to the use of said supports for performing probe-based assays and to a kit of parts comprising said support and a detection means.

The present invention specifically relates to a method for the preparation of metal oxide supports loaded with biomolecules, comprising the steps of: (a) activating the surface of the support by means of an silanating agent comprising an amine group; and (b) loading the support by attaching biomolecules to the activated surface.

Such a method is known, e.g. from WO 99/002266, in which method aluminium oxide membranes are activated using 3-aminopropyl triethoxysilane (APS) after which oligonucleotide probes are covalently coupled to the activated membranes. A similar method is described for aluminiumoxide nanoparticles carrying antigens (Bioconjugate Chem. **1997, 8,** 424-433). Further, for chromatographic purposes, aluminium oxide supports were used for covalent immobilization of concavalin A, after previous activation with APS (Biotechnology and Applied Biochemistry **16,** 221-227 (1992)). Balladur et al., J. of Colloid and Interface Science, 194, 408-418, 1997 describe the adsorption of oligonucleotides on aminopropyl silane-modified silica wafers. These and similarly loaded metal oxide supports are thus useful for instance in probe-based assays, further for carrying biomolecules, e.g. for use as vaccines, and for separating other substances from mixtures by hybridizing, binding or interacting otherwise with those other substances.

A disadvantage of such supports is that a number of amino-groups of the silanating agent comprising an amine group used for the activation of the metal oxide surface, are still present as unloaded amino-groups even after the support has been loaded with the appropriate biomolecule. This may result in unwanted interactions (non-specific or a-specific interactions) of these amino-groups with various substances present in the medium in which the loaded support is used. For example, when used in probe-based assays, such a-specific interactions may generate high background signals disturbing the signals of the analyte, bound to the capture-biomulecules.

It has now been found that when the method of the invention is applied, the non-loaded amino-groups are removed selectively from the surface of the further loaded support without affecting the loaded part of the surface.

The invention thus relates a method for the preparation of metal oxide supports loaded with biomolecules, comprising the steps of: (a) activating the surface of the support by means of a silanating agent comprising an amine group; (b) loading the support by attaching biomolecules to the activated surface, characterized in that subsequently the loaded support is treated with an acidic solution, and provided that the method is not used for the preparation of silica wafers which are aminated by silanation using (3-aminopropyl)monoethoxydimethylsilane, and loaded with oligonucleotides. Such loaded silica wafers have been subject to a study regarding the effect of pH on the maximum adsorbed amount of oligonucleotides onto the aminated silica wafer [Balladur et al., J. of Colloid and Interface Science, 194, 408-418, 1997]. Therefore this invention does not relate to (a method for the preparation of) such silica wafers.

According to another embodiment of the invention the activated and loaded support is subsequently treated with a basic solution (preferably for a prolonged period of time), provided that the method is not used for derivatization of aluminiumoxide nanoparticles aminated with (3-aminopropyl)triethoxysilane, wherein the basic solution further contains a large excess of N-acetylhomocysteinelactone. Such derivatized aluminiumoxide nanoparticles were prepared without the intention to remove aminogroups (Bioconjugate Chem. 1997, 8, 424-433) and are not an embodiment of the present invention (nor is the method for their preparation).

According to another embodiment of the invention the activated and loaded support is subsequently treated with a neutral solution (preferably for a prolonged period of time).

The usual methods for suppressing a-specific interactions of unloaded amino-groups on different types of supports involve chemical "capping" / "blocking" the unloaded amino-groups or prehybridizing them with DNA (see for example: Science, Vol. 270, 1995, 467-470; Nucleic Acids Research, Vol. 15 (13) 1987, 5353--5373; Nucleic Acids Research, Vol. 26 (17) 1998, 3883-3891, etc.). However, those methods are not very well defined and require more difficult procedures. Furthermore, with those methods the unloaded amino-groups are not actually removed from the surface, which is the case when the method of the present invention is applied.

The method of the invention is well defined and easy to perform and the results, e.g. in terms of signal/background ratio's, are comparable or even better when compared to the usual procedures.

In any situation in which unloaded amino-groups are to be selectively removed from metal oxide supports loaded with biomolecules, the method of the present invention is applicable, provided that a silanating agent comprising an amine group has been used to activate the metal oxide surface.

The pH value of the solution with which the loaded supports may be treated according to the invention, depends substantially on the type of biomolecule attached to the surface and on the way of attaching (loading) the biomolecules to the surface, either covalently or adsorptively.

When the biomolecule is attached to the surface covalently (i.e. they are attached to the amino-groups on the surface via some chemical linkage) and it can withstand both acidic and basic conditions, both acidic and basic solutions may be used for the removal of unloaded amino-groups. If the biomolecule is sensitive to either acidic or basic conditions, respectively a basic or acidic solution must be chosen for the removal of the unloaded amino-groups.

When adsorptively attached (i.e. the biomolecule adheres to the the surface modified with a silanating agent comprising an amine group), also the overall charge of the biomolecule is a relevant parameter. For example, when a negatively charged biomolecule is adsorptively attached to the activated surface of the metal oxide support, it may become detached at a certain pH when the charge of either the biomolecule is reduced by protonation or the charge of the surface is reduced due to deprotonation. For example, an oligonucleotide having a negatively charged backbone will be protonated at very low pH values and it will subsequently be released from the positively charged activated surface of the support. Similarly, the loaded amino-groups are deprotonated at very high pH values (e.g. 11.5) resulting in the release of the negatively charged oligonucleotide.

A person skilled in the art will know how to find the balance between effective removal of the unloaded amino-groups and the intactness of the loaded support. Some preliminary experimentation may be required to find the optimal conditions for the specific circumstances. However, use of an acidic solution is preferred, since the removal of unloaded amino-groups

takes place much more rapidly than in basic conditions. At very low pH values it may even occur almost instantaneously (if the circumstances allow to use such a solution). Preferred are solutions with a pH 2 to 7. For surfaces to which oligonucleotides are attached a solution of pH 4-5 is particularly preferred.

The treatment with a neutral or basic solution is also effective, but requires longer treatment periods. The applicable pH range for a certain situation depends on the reagent used to coat the surface. The pKa of the amine groups influences the charge on the surface and thus the pH at which biomolecules, especially adsorbed biomolecules, stay attached to said surface. Of course the charge distribution on the biomolecules is also important. As explained above, depending on their negative charge, biomolecules may become detached when the pH is very low. The treatment may take several hours up to about a day, depending on the pH value of the solution, the type of metal oxide of the support and the type of reagent used to activate the surface of the support.

The acid- or base-treatment time is not very critical in terms that the performance of the loaded support is not significantly affected after long treatment periods. Also the temperature at which the method may be performed is not very critical. However, temperatures up to 40 °C are preferred. Most preferably the method is applied at room temperature (18 - 24 °C).

Suitable (acidic or basic) solutions for use in the method according to the invention are aqueous buffered solutions adjusted to the desired pH value, for example, but not limited to, for acidic solutions: acetate buffers, phosphate buffers, citric acid buffers, and the like, for basic solutions: disodium hydrogen phosphate or sodium phosphate buffers, sodium tetraborate buffers, tris-buffers, diethanolamine/hydrogen chloride buffers, carbonate buffers, and the like. Appropriate buffers have a concentration of 40 mM, but also other concentrations may very well be applicable.

Metal oxide supports which may be used in the method of the invention are supports of metal oxides of, for example and not limited to, tantalum, titanium and aluminium, silicium as well as alloys of two or more metals and doped metals and alloys. Particularly useful supports are (electrochemically manufactured) porous metal oxide membranes. A preferred metal oxide is aluminium oxide. Preferred porous metal oxide membranes are the membranes mentioned in WO 99/02266.

The surface of the metal oxide support may be activated using different types of silanating agent comprising an amine group. Effective activating reagents are for example 3-aminopropyl triethoxysilane, 4-aminobutyl-dimethyl-methoxysilane, 3-[2-(2-aminoethylamino)ethylamino]-propyltrimethoxysilane, 3-(2-aminoethylamino)propyl-methyldimethyoxysilane, 3-(2-amino-ethylamino)propyltrimethyoxysilane, 3-aminopropyl-methyl-diethoxysilane, (3-aminopropyl)-tris[2-(2-methoxyethoxy)ethoxy]silane and 4-aminobutyltriethoxysilane, however also other silanating agents comprising an amine group may be equally suitable. A preferred silanating agent is aminopropyl triethoxysilane (APS) used in an unbuffered aqueous solution. The silanation reaction may be performed in aqueous solution, in organic solution or in the gas phase.

Biomolecules may be attached to the support either covalently or adsorptively. In a preferred embodiment of the invention the biomolecules are adsorptively attached to the activated surface of the support. Preferably, the biomolecules are attached to the surface in spots, thereby forming a (micro)array of spots. A preferred method to spot the surface with biomolecules applies inkjet technology. This technology allows for the accurate deposition of defined volumes of liquid. (See e.g. T.P. Theriault: DNA diagnostic systems based on novel Chem-Jet technologies, IBC Conference on Biochip Array Technologies, Washington DC, May 10, 1995)

In a highly preferred embodiment of the invention an acidic solution of pH 5 is used for the preparation of APS-activated aluminium oxide porous membranes (which are electrochemically manufactured), spotted with arrays of oligonuceotides, which oligonucleotides are adsorptively attached to the activated surface.

Metal oxide supports prepared according to the method of the invention, are very useful for diagnostic purposes, for example in probe-based assays. In particular (micro)arrays, on the surface of which essentially no free amino groups are present that lead to a-specific binding, are very suitable for that purpose. In such arrays, the density of amino groups on the surface area between the spots is significantly lower than the density of amino groups in the spots. Preferably, the arrays comprise different biomolecules in different spots, allowing multi-analyte

detection. An analyte is a substance, usually a biomolecule, in a mixture which may be detected because of its capability to interact specifically with a selected reagens (for example another biomolecule capable of reacting with the analyte) (on a support).

Probe-based assays comprise for example nucleic acid hybridization assays and immunological assays. In such assays, a sample which comprises an analyte is contacted with a loaded support prepared according to the invention. The analyte is subsequently allowed to bind to the biomolecule which is attached to the surface of the support. Detection of binding can be performed by (1) adding a detection means, for example a substance capable of binding to the analyte, which substance is provided with a label, (2) allowing the detection means to bind to the complex of the analyte and the biomolecule, and (3) determining whether the label is present at the position where the biomolecule was attached. Alternatively, the analyte may already have been provided with a label, in which case binding to the biomolecule can be detected directly, without the addition of a detection means.

The present invention therefore also relates to a kit of parts comprising a metal oxide support according to the invention, further comprising a detection means for determining whether binding has occurred between the biomolecules and an analyte. Preferably, such detection means is a substance capable of binding to the analyte and being provided with a label. Such label is in particular useful, if it is capable of inducing a colour reaction and/or capable of bio- or chemo- or photoluminescence.

Biomolecules which may be used in the process of the invention include oligonucleotides and other negatively charged capture molecules, such as antibodies, antigens, peptides, receptors, haptens and ligands for receptors (which may be modified to introduce (additional) negative charges). Preferred are oligonucleotides. However, the scope of the invention is not limited to these specific molecules. The method may very well be useful to supports loaded with other types of molecules as well.

The invention is further illustrated by the following examples.

EXAMPLES

Preparation of APS-membranes:

Membranes (Anodisc 25, 0.2 um; Whatman) were silanated by placing them in a 1 % solution of 3-aminopropyltriethoxysilane (Acros, APS) in demineralised water (milliQ), for a period of 1 hr on a plateshaker. The membranes were soaked in water to remove the bulk of the APS molecules and subsequently washed individually by sucking 5 ml of milliQ through the membranes to remove the last traces of free APS. These membranes were heated for 2 hr's at 120 °C under vacuum. After cooling, these "APS-membranes" were stored in a vacuum exsiccator, under argon over KOH until they were used.

Stability study of APS-membranes:

The following buffers were made:

- 40 mM Acetate buffer pH 5.0; from sodiumacetate and acetic acid
- 40 mM Acetate buffer pH 6.0; from sodiumacetate and acetic acid
- 40 mM Phosphate buffer pH 7.0; from sodiumdihydrogenphosphate and NaOH
- 40 mM Borate buffer pH 8.0; from sodium tetraborate and hydrogen chloride
- 40 mM Borate buffer pH 9.0; from sodium tetraborate and hydrogen chloride

The APS membranes were incubated at the above mentioned pH's using the appropriate buffer for a period between 0.25 and 22 hr's (see table 1, for details). After this period the membrane was removed from the buffer solution and washed with milliQ and ethanol and dried under vacuum at room temperature. Subsequently the amount of amino-groups was determined using a modified trinitrobenzenesulfonic acid (TNBS) method [Habeeb, A.F.S.A. (1996), Anal. Biochem. **14**, 328. See also Sashidhar, R.B., Capoor, A.K., Ramana, D., (1994), J. Immumol. M]. A commercially available stock solution (Sigma) of 5% (m/v) of TNBS was diluted 25-fold with a 50 mM borate buffer of pH 10.0 directly before the amino-group determination was performed. Each treated membrane was placed individually in a beaker and 4 ml of the diluted

TNBS solution was added and the membrane was incubated on a plateshaker for 1 hr at room temperature. The yellow/orange membranes were thoroughly washed (by using vacuum to suck liquids through the membrane) with milliQ to remove excess TNBS. Subsequently the membranes were dissolved in a known amount (e.g. 4.0 ml) of a 4.0 M NaOH solution in milliQ. After filtration of this solution over a 0.45 µm filter, the absorption was measured using an UV/vis spectrophotometer. Using the extinction coefficient of the adduct at 390 nm of 11929 l/mol/cm, the number of amino groups per membrane that reacted with TNBS could be determined. In table 1 the decrease (in %) in amino-groups as function of time and pH is given.

Time (hr)	Percentage of aminogroups at				
	pH 5.0	pH 6.0	PH 7.0	pH 8.0	pH 9.0
Blanc exp.	100	100	100	100	100
0.25	54				
0.5	19				
1	9				
1.5		49			
2.5		17	82	96	51
6			69	52	34
22			54	42	21

Table 1: degradation as function of pH and time of APS membranes

From this table it is clear that the loss of aminogroups is fastest at lower pH's.

Protective effects of loading of the activated surface of a support:

(a) Protective effect of the TNBS group:

In another experiment the aminogroups on an APS membrane reacted with TNBS as described above. This yielded membranes that contain covalently linked chromophores that consist of the trinitrobenzene adduct of the amino group (APS-TNB membranes).

These membranes were treated with the same buffers mentioned above for a period of 22 hr's at room temperature. In table 2 the remaining amount of chromophores after this period, as determined with the above mentioned method, are summarized.

pH applied	Resulting aminogroups cq chromophores (%)
Blanc experiment	100
5.0	71
6.0	95
7.0	83
8.0	90
9.0	95

Table 2: stability of TNB-APS membranes

This table clearly shows, that functionalisation of the aminogroups results in a large increase of the stability. In other words, the majority of the functionalized aminogroups attached to the surface is not affected.

(b) Protective effect of DNA

A haring sperm DNA solution of 1 µg/ml in milliQ was prepared and 1 µl of this solution was pipetted onto the APS membranes in spots. These membranes were devided in two sets: one which was treated with pH 5 buffer (for 1 hr) and the other which were not. Both sets of membranes were subsequently treated with TNBS (method described above) in order to detect any decrease in amino-groups. This yielded the following results. The total surface of the untreated membrane showed the expected orange colour since no degradation (removal) of APS groups had taken place. The surface of the membrane that was treated at pH 5.0 was not orange but very slightly yellow, due to the degradation of nearly all APS molecules. However, at the spots where the DNA was pipetted on the membrane, an orange colour was still visible, indicating the protective effect of the DNA on the degradation process of the aminogroups on the surface. It was shown in a blanc experiment that the amino groups of DNA did not react

with TNBS in such a way that this could explain the orange colour observed for the pH 5 treated membrane.

(c) Protective effect of oligonucleotides

A solution of 200uM oligonucleotide with the sequence 5'TAT GGC TCT CCC GGG AGGGGG GGT CCT GGA 3' was prepared. This solution was pipetted onto two unmodified membranes and onto two APS membranes. In this process only one half of every membrane was covered with the oligonucleotide. Subsequently these membranes were dried at 37 °C for one hr. One unmodified and one APS membrane were treated with pH 5 solution as described above. Subsequently all membranes were washed with phosphate buffer pH 7.4 and ethanol and dried. All membranes were placed in the diluted TNBS solution as described above and incubated for one hr. After washing and drying, the unmodified membranes were totally white, while the total surface of the APS membrane that was not treated with pH 5 was yellow. The pH 5 treated APS membrane showed a slightly yellow color on the half that was covered with the oligonucleotides while the other half was totally white.
This experiments clearly shows the protective effect of oligonucleotides.

(d) Arrays of oligonucleotides:

The protective effect was also visualised in a different way using hybridization of oligonucleotides:
Using a commercially available spotter (Packard instruments), droplets of 300 picoliter of oligonucleotide A (100 µM dissolved in milliQ) were spotted onto APS membranes:
This oligonucleotide A is defined by the sequence: 5'-TTG TAC AGA ACT GGA AAA GGA 3'. Also available for hybridization experiments is oligonucleotide B (sequence 5'-TCC TTT TCC AGT TCT GTA CAA 3') which is complementary to A and labeled with a fluoresceine moiety at the 5' end.

After spotting, all membranes were dried at 50 °C for 1 hr. One set of membranes was incubated in an acetate buffer (40 mM, pH 5.0) for 30 minutes and subsequently washed with

milliQ, ethanol, and dried under vacuum. These membranes will be referred to as "pH 5 arrays". As a blanc experiment the other set of membranes was not treated with the pH 5.0 solution, but it was only washed with milliQ, ethanol and dried under vacuum. These membranes will be called "blanc array". All membranes were stored under argon untill being used for hybridisation experiments.

The membranes were placed in a homebuilt device that allows 25 µl of a phosphate buffer (pH 7.0) containing the complementary oligonucleotide **B** being flushed back and forth through the membrane. If the solution is below this device, a hybridised nucleotide on the membrane can directly be seen due to its fluorescent label using an ordinary fluorescence microscope and a CCD-camera. With this setup, the data in table 3 have been collected. One cycle is defined as the 25 µl being pumped up and down one time through the membrane. Specific signals have been corrected for the background signal.

	Signal (arb.units) for pH 5 arrays			Signal (arb.units) for blanc arrays	
Cycles	Specific	Background	Cycles	Specific	Background
0	0	5	0	0	5
2	4	8	1	0	41
5	7	8	5	2	47
10	11	9			
15	13	9			
20	15	9			

Table 3: Specific and a-specific interactions of pH 5 arrays and blanc arrays

From this table the following observations are important:

- The background signal for pH 5 arrays is low and nearly constant
- The specific hybridisation for pH 5 arrays takes place rapidly and can easily be monitored

- The blanc array does not show specific signals, due to a strong a-specific interaction between the amino-groups (which are present all over the membrane) and oligonucleotide B.
- The a-specific interactions (background) that are seen for the blanc arrays, is highly reduced by the acidic treatment.

Thus, this experiment shows that the acidic treatment removes all amino groups that are not covered and protected by oligonucleotides. Oligonucleotide A is not removed from the surface indicating that it is still captured by aminogroups on the surface below the nucleotides which have not been removed.

CLAIMS

1. A method for the preparation of metal oxide supports loaded with biomolecules, comprising the steps of :

(a) activating the surface of the support by means of a silanating agent comprising an amine group;

(b) loading the support by attaching biomolecules to the activated surface,

characterized in that subsequently the loaded support is treated with an acidic solution, and provided that the method is not used for the preparation of silica wafers which are aminated by silanation using (3-aminopropyl)monoethoxydimethylsilane and loaded with oligonucleotides.

2. A method for the preparation of metal oxide supports loaded with biomolecules, comprising the steps of :

(a) activating the surface of the support by means of a silanating agent comprising an amine group;

(b) loading the support by attaching biomolecules to the activated surface,

characterized in that subsequently the loaded support is treated with a basic or neutral solution, and provided that the method is not used for derivatization of aluminiumoxide nanoparticles aminated with (3-aminopropyl)triethoxysilane, wherein the basic solution further contains a large excess of N-acetylhomocysteinelactone.

3. The method of claim 1, wherein the solution is of pH 2 to 7 .

4. The method of claim 3, wherein the biomolecules are oligonucleotides and the pH is 4-5.

5. The method of claim 1, wherein the metal oxide support is a (electrochemically manufactured) porous metal oxide membrane.

6. The method of claim 5, wherein the metal oxide is aluminium oxide.

7. The method of claim 1, wherein the support is activated by means of a silanating agent comprising an amine group selected from 3-aminopropyltriethoxysilane, 4-aminobutyl-dimethyl-methoxysilane, 3-[2-(2-aminoethylamino)ethylamino]propyl-trimethoxysilane, 3-(2-aminoethylamino)propyl-methyldimethyoxysilane, 3-(2-aminoethylamino)propyl-trimethyoxysilane, 3-aminopropyl-methyl-diethoxysilane, (3-aminopropyl)tris[2-(2-methoxyethoxy)ethoxy]silane and 4-aminobutyltriethoxysilane,

8. The method of claim 7, wherein the silanating agent comprising an amine group is 3-aminopropyltriethoxysilane.

9. The method of claim 8, wherein 3-aminopropyl triethoxysilane is used in an unbuffered aqueous solution.

10. The method of claim 1, wherein the biomolecules are adsorptively attached to the activated surface of the support.

11. The method of claim 1, wherein the biomolecules are attached to the activated surface in spots, thereby forming an array of spots.

12. The method of claim 11, wherein the biomolecules attached to the surface in different spots may be the same or different.

13. The method of claim 1, wherein the biomolecules are oligonucleotides.

14. A loaded metal oxide support prepared according to the method of claim 1.

15. An aminoalkyltrialkoxysilane-activated metal oxide support, provided with an array of spots of biomolecules attached to the support, characterized in that on the array the density of amino groups on the surface area between the spots is significantly lower than the density of amino groups in the spots.

16. Use of the metal oxide support of claim 14 or 15 for performing a probe-based assay.

17. A kit of parts comprising the metal oxide support of claim 14 or 15, further comprising a detection means for determining whether binding has occurred between the biomolecules and an analyte.

18. A kit according to claim 17, wherein the detection means is a substance capable of binding to the analyte and being provided with a label.

19. A kit according to claim 18, wherein the label is capable of inducing a colour reaction and/or capable of bio-, chemo- or photoluminescence.

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(54) Title: PREPARATION OF METAL OXIDE SUPPORTS LOADED WITH BIOMOLECULES

(57) Abstract: The invention relates to method for the preparation of metal oxide supports loaded with biomolecules, comprising the steps of: (a) activating the surface of the support by means of a silanating agent comprising an amine group; (b) loading the support by attaching biomolecules to the activated surface, characterized in that subsequently the loaded support is treated with an acidic solution, and provided that the method is not used for the preparation of silica wafers which are aminated by silanation using (3-aminoprdeyl)monoethoxydimethylsilane and loaded with oligonucleotides. Similarly, an activated and loaded support may be treated with a basic or neutral solution, provided that the method is not used for derivatization of aluminiumoxide nanoparticles aminated with (3-aminopropyl)triethoxysilane, wherein the basic solution further contains a large excess of N-acetylhomocysteinelactone. This method can for example be used in the preparation of arrays for probe-based assays.

PTO/SB/01 (10-01)
Approved for use through 10/31/2002. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☑ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	65959/16
First Named Inventor	F. Venema
COMPLETE IF KNOWN	
Application Number	10/049,804
Filing Date	February 14, 2002
Art Unit	to be assigned
Examiner Name	to be assigned

As the below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original and first inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PREPARATION OF METAL OXIDE SUPPORTS LOADED WITH BIOMOLECULES

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☑ was filed on (MM/DD/YYYY) 02/14/2002 as United States Application Number or PCT International Application Number 10/049,804 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or (f), or 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	Certified Copy Attached? NO
PCT/EP00/07736	PCT	08/09/2000	☐	☐	☑
EP 99202649.2	Europe	08/16/1999	☐	☐	☑
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

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DECLARATION — Utility or Design Patent Application

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Given Name (first and middle [if any]): F. | Family Name or Surname: Venema

Inventor's Signature: [signature] | Date: 17th June 2002

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NAME OF SECOND INVENTOR: ☐ A petition has been filed for this unsigned inventor

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Inventor's Signature | Date

Residence: City | State | Country | Citizenship

Mailing Address

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☐ Additional inventors are being named on the ____supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

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POWER OF ATTORNEY OR AUTHORIZATION OF AGENT

Application Number	10/049,804
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First Named Inventor	F. Venema
Title	Preparation of Metal Oxide Supports Loaded with Biomolecules
Group Art Unit	Not assigned
Examiner Name	Not assigned
Attorney Docket Number	65959/16

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☑ Applicant/Inventor.

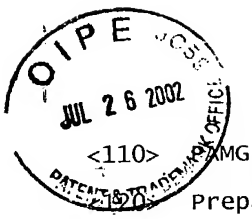
☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

SIGNATURE of Applicant or Assignee of Record

Name	F. Venema
Signature	
Date	17th June 2002

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☑ *Total of 1 forms are submitted.



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